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## **Comparison of Nine Platelet Function Tests Used to Determine Responses to Different Aspirin Dosages in People with Type 2 Diabetes**

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## Abstract

The antiplatelet efficacy of aspirin (ASA) is reduced in type 2 diabetes (T2D). As the best ex-vivo method of measuring ASA efficacy remains uncertain, we compared nine platelet function tests to assess responsiveness to 3 ASA dosing regimens in 24 T2D patients randomized in a 3-treatment crossover design to ASA 100 mg/day, 200 mg/day or 100 mg twice daily for 2-week treatment periods. Platelet function tests compared were: Light transmission aggregometry (LTA-0.5mg/ml of arachidonic acid (AA) and 10  $\mu$ M ADP); Multiplate whole blood aggregometry (WBA-0.5mM AA and 6.5 $\mu$ M ADP); PFA-100<sup>TM</sup>-CADP and CEPI; VerifyNow<sup>TM</sup>-ASA; Urinary 11-dehydro-thromboxane B2 (TxB<sub>2</sub>) and Serum TxB<sub>2</sub>. All cyclo-oxygenase (COX-1) dependent tests and some COX-1 independent tests (PFA-CEPI, LTA-ADP) demonstrated significant reductions in platelet reactivity with all ASA doses. Two COX-1 independent tests (WBA-ADP and PFA-CADP) showed no overall reduction in platelet reactivity. Overall classifications for detecting all ASA doses, compared to baseline, were: Very good-LTA-AA ( $k=0.95$ ) and VerifyNow<sup>TM</sup>-ASA ( $k=0.85$ ); Good-Serum TxB<sub>2</sub> ( $k=0.79$ ); Moderate-LTA-ADP ( $k=0.59$ ), PFA-100<sup>TM</sup>-CEPI ( $k=0.56$ ), Urinary TxB<sub>2</sub> ( $k=0.55$ ), WBA-AA ( $k=0.47$ ); Poor-PFA-100<sup>TM</sup>-CADP ( $k=-0.02$ ) and WBA-ADP ( $k=-0.07$ ). No significant kappa statistic differences were seen for each test for each ASA dose. Correlations for each test with serum TxB<sub>2</sub> measurements were: Very good - VerifyNow<sup>TM</sup>-ASA ( $k=0.81$ ,  $R^2=0.56$ ), LTA-AA ( $k=0.85$ ,  $R^2=0.65$ ); Good-PFA-100<sup>TM</sup>-CEPI ( $k=0.62$ ,  $R^2=0.30$ ); Moderate-Urinary TxB<sub>2</sub> ( $k=0.57$ ,  $R^2=0.51$ ), LTA-ADP ( $k=0.47$ ,  $R^2=0.56$ ); Fair-WBA-AA ( $k=0.31$ ,  $R^2=0.31$ ) and Poor-PFA-100<sup>TM</sup>-CADP ( $k=0.04$ ,  $R^2=0.003$ ), WBA-ADP ( $k=-0.04$ ,  $R^2=0.0005$ ). The platelet function tests we assessed were not equally effective in measuring the antiplatelet effect of ASA, and correlated poorly amongst themselves but COX-1 dependent tests performed better than non-COX-1 dependent tests.

## Introduction

Reduced efficacy of aspirin (ASA) to inhibit platelet aggregation is observed frequently in type 2 diabetes (T2D), compared with the general population, and has been referred to as “ASA resistance” or hypo-responsiveness [1-3]. Failure to respond to ASA therapy is normally a result of a combination of factors including genetic polymorphisms, drug interactions and poor compliance [4, 5]. Despite this, current guidelines do not recommend laboratory testing for monitoring and personalising ASA therapy [6]. T2D is a pro-thrombotic state characterized by increased platelet reactivity and turnover, increased hepatic synthesis of fibrinogen and plasminogen activator inhibitor (PAI-1) and quantitative changes in the glycation and oxidation of clotting factors [1, 7, 8]. Accordingly, it is possible that higher or more frequent doses of ASA might be required to potentially overcome metabolic barriers in T2D, particularly in conditions where platelet turnover is increased [9]. Once-daily low dose ASA dosing may be, therefore, insufficient when platelet turnover is increased as irreversibly inhibited platelets are removed more rapidly from the circulation and replaced by new platelets not yet exposed to ASA [9-11]. Enteric coatings of ASA have also been shown to be an important variable for contributing to reduced bioavailability and poor responsiveness to ASA in T2D [12]. Twice daily dosing has also been advocated for treating patients with essential thrombocythemia as well as T2D where there is evidence of increased platelet turnover [13, 14].

Our recent study demonstrated that ASA 100 mg given twice daily was more effective in reducing platelet reactivity than 100 mg given once daily in T2D, despite showing that there was no evidence of increased platelet turnover in these patients [15]. The study primary objective was to compare changes from baseline in platelet reactivity with each of three ASA regimens, as measured by the VerifyNow™-ASA point-of-care test (POC) (Accumetrics, CA, USA)[16, 17]. Secondary objectives were to further assess changes in platelet function using a variety of both specialised and point-of-care COX-1 dependent/independent platelet function tests. Many tests are now available for measuring inhibition of platelet function but they are becoming increasingly diverse and simpler to perform [18]. Although light transmission aggregometry (LTA) is still regarded as the gold standard despite being invented in the 1960's, poor standardization and its restriction to specialised laboratories means that the test is limited for potential clinical utility [18]. Measurement of thromboxane and its

metabolites within serum and urine are important for specifically measuring the biochemical consequences of ASA inhibition of platelet COX-1 activity but again are both restricted to specialised laboratories. In the last 20 years a number of simpler semi and full point-of-care tests have been developed (e.g. PFA-100™, Multiplate and VerifyNow™) but it is still unclear whether these tests are actually useful for monitoring ASA in this context and how they may perform against LTA and Thromboxane measurements [18-20]. As we previously demonstrated that there were clear differences in the ability of some of these tests to detect differences between the 3 ASA dose regimens [15], the aim of this additional study was therefore to not only compare and contrast but examine in detail the performance characteristics of all these tests in each of the three ASA regimens used in the trial.

## **Materials and Methods**

This investigator-led, single-centre, randomized, prospective, double-blind study received ethical approval from the South East London Research Ethics Committee 3 [15]. It complied with The World Medical Association Declaration of Helsinki (1964) and its amendments and clarifications, the European Union Clinical Trials Directive (2001/20/EC), and International Conference on Harmonisation Harmonized Tripartite Guideline for Good Clinical Practice (CPMP/ICH/135/95). All participants provided written informed consent. The authors are solely responsible for the design, conduct, and analysis of this study, the drafting and editing of the manuscript, and its final contents.

## ***Participants***

Twenty-four patients with T2D were recruited from local primary care clinics and from the Oxford Centre for Diabetes, Endocrinology, and Metabolism research recruitment register. Eligible participants were required to be at least 18 years of age, have no prior history of cardiovascular disease, be on stable diabetes therapy with diet or oral anti-hyperglycemic agents for at least 3 months, with HbA1c < 10% (86 mmol/mol) and triglycerides ≤2 mmol/L. Patients with the following conditions were excluded: pregnant or lactating; peptic ulcer disease or other gastro-intestinal disorder; blood pressure >150/100 mmHg; bleeding disorder; evidence of severe hepatic disease or ALT>3-times the upper limit of normal; severe

renal disease or eGFR  $<40\text{ml/min/1.73m}^2$ ; currently taking or with a contraindication to treatment with aspirin.

### ***Study design***

In this 3-treatment, crossover study, participants were randomized to one of six possible treatment sequences during which they received the identical formulation of ASA (acetylsalicylic acid, Bayer Pharma AG, Germany) or matching placebo as two tablets twice a day according to 3 different regimens with active therapy ; 100 mg once-daily, 200 mg once-daily or 100 mg twice-daily. Each 2-week treatment period was followed by a 2-week washout [15]. ASA doses were chosen to approximate doses used in clinical practice and to facilitate the use of a double-blinded placebo control.

### ***Blood Sampling***

Fasting venous blood and urine samples were taken at baseline, and then on completion of each treatment period. All samples were taken between 8 am and noon and 12 or 24 hours after the ingestion of the last ASA dose (depending upon the dosage regime) to minimize the immediate impact of ASA administration and circadian effects on platelet function. Samples were transported to the local platelet laboratory by hand in upright tubes in a rack kept at room temperature, with platelet function testing performed  $> 30$  minutes but within 4 hours of collection in keeping with British Committee for Standards in Haematology guidelines for platelet function testing. All samples were coded and blinded for ASA dosages. Blood samples for platelet function (LTA, PFA-100<sup>TM</sup> and WBA) were anticoagulated with 1/10 volume of 3.2% trisodium citrate in vacutainers (Becton Dickinson, Oxford UK). As recommended the 2 mL Greiner Bio-One partial-fill vacuette tube with 1/10 volume of 3.2% sodium citrate was used for blood samples to be tested on the VerifyNow<sup>TM</sup>-ASA. Serum samples were clotted at 37°C with a standardised incubation of 30 minutes before centrifugation at 2000 g for 20 minutes and the serum stored at -70°C until analysis. Stabilized urine samples were collected into universal tubes (25 ml) and stored in aliquots at 70°C until analysis. EDTA vacutainers were used for blood counting.

### ***Platelet Function Testing***

The VerifyNow™-ASA (Accumetrics, CA, USA) test was selected as being the most convenient method for assessing the impact of ASA on platelet reactivity in this trial. The primary objective was therefore to compare changes from baseline in platelet reactivity with each of three ASA regimens [15]. The VerifyNow™-ASA Point-of Care test Cartridges were stored at 4°C and warmed up to room temperature before analysis. The same batch of cartridges was used for the entire trial. Internal quality control of the instrument was performed on each day of testing and test cartridges were then loaded into the instrument immediately followed by insertion of the mixed special citrated whole blood sample onto the needle within the cartridge to initiate the test. The instrument then withdraws the blood under vacuum into the various reaction channels of the cartridge where automated LTA is then performed using arachidonic acid as the agonist. After the test has finished the results were displayed and printed. The results of this test, which is based on turbidimetric optical detection of platelet aggregation in whole blood, are expressed in ASA reactive units (ARUs), with a lower limit of normal (LLN) >550 ARU. Secondary objectives were to further assess changes in platelet function using a variety of COX-1 dependent and COX-1 independent platelet function tests. Light transmission aggregometry (LTA) was measured with a PAP-4 light transmission aggregometer (Biodata corporation) with 0.5 mg/mL arachidonic acid (AA) and 10 µM adenosine diphosphate (ADP) as agonists with unadjusted Platelet Rich Plasma (PRP) counts in line with recent guidelines. PRP was prepared by centrifugation of citrated whole blood by centrifugation at 200 g for 10 minutes and stored at room temperature in capped tubes. Platelet Poor Plasma (PPP) was prepared by centrifugation at 2000 g for 20 minutes. 270 µl of PRP and PPP was pipetted into PAP-4 aggregometry tubes and a magnetic stir bar added to the PRP tube and the tubes incubated at 37°C for 5 minutes before testing. In each channel, 100% maximal aggregation was set with PPP and then a 0% baseline established with PRP for at least 2 minutes before addition of 30 µl of stock agonists. The same source and batch of each agonist were used for the entire study and stored frozen at -70°C. Arachidonic acid was stored frozen at 5 mg/ml in distilled water in glass vials. Before testing agonists were thawed and then kept on ice. Results were reported as maximal platelet aggregation after 10 minutes with LLN ≥20% for AA and ≥70% for ADP. Multiple electrode whole blood aggregometry (WBA) was performed using a 5-channel Multiplate™ aggregometer (Roche Diagnostics, Munich, Germany) with an integrated computer and guided automatic pipetting. Citrated whole blood samples were mixed gently before pipetting into disposable ready to use test cells containing

two independent sensor units consisting of two silver coated highly conductive copper electrodes and a stir-bar. In each test cuvette, mixed whole blood was immediately diluted 1:1 vol/vol with 0.9% saline solution at 37°C for 3 minutes before addition of 30 µl of each agonist. Final concentration of agonists were 0.5 mM AA and 6.5 µM ADP as provided by the manufacturer. Results are expressed as area under the curve (AUC) with LLN >71 for AA and >57 for ADP as provided by the manufacturer. The platelet function analyzer (PFA-100™, Siemens Diagnostics, IL, USA) used either collagen and epinephrine (CEPI) or collagen and adenosine diphosphate (CADP) cartridges to measure platelet thrombus formation under high shear. Cartridges were stored at 4°C and warmed up to room temperature before testing commenced and the same batch used for the entire trial. A daily quality control instrument check was also performed on days of testing. 0.8 ml of citrated whole blood was pipetted into the sample reservoir each cartridge to initiate the test. Results were reported as closure times (CT) in seconds with upper limit of the normal range >164 seconds for CEPI and >112 seconds for CADP. Maximal CTs were recorded as >300 seconds. Pharmacologic effects of ASA were assessed by measuring serum thromboxane B<sub>2</sub> (TxB<sub>2</sub>) and urinary 11-dehydrothromboxane B<sub>2</sub> (dTxB<sub>2</sub>). Serum TxB<sub>2</sub> was assayed by ELISA (R&D systems, Abingdon, UK) according to the manufacturer's instructions with LLN 10 ng/mL. Urinary dTxB<sub>2</sub> was measured using ELISA (AspirinWorks Test kit, Corgenix, Peterborough, UK) according to the manufacturer's instructions with LLN ≥1500 pg/mg urinary creatinine. For both assays all samples were diluted appropriately so that the optical densities were within the standard curve. Creatinine levels were measured in the pathology department at the John Radcliffe Hospital in Oxford.

### ***Statistical Analysis***

Sample size estimates based on previous VerifyNow™-ASA Point-of Care studies suggested that 17 patients would be required to detect a clinically-significant 27% effect size between any two ASA regimens, with 90% power at the 5% significance level. A total of 24 patients were recruited to ensure balanced allocation to the 6 possible treatment sequences, and to allow for potential dropouts. The results of all platelet function tests were compared at baseline and after each ASA regimen using kappa statistics. Each platelet function test was also compared with Serum TxB<sub>2</sub> (as a measure of the direct pharmacological action of ASA)



and Arachidonic Acid stimulated LTA (the perceived gold standard of platelet function testing) by kappa statistics and Spearman correlations.

## Results

### *Participant Characteristics*

Participant baseline characteristics are summarized in the primary outcome paper [15]. Briefly, participants were mean ( $\pm 1SD$ ) age  $51 \pm 7$  years, BMI  $31.4 \pm 7.2$  kg/m<sup>2</sup> with a median duration of diabetes of 2 years. Nineteen participants were treated with metformin, 6 with a sulfonylurea and 2 with a thiazolidinedione. None were treated with other glucose-lowering therapies at any time during the study.

For the primary objective, data from two of the 72 treatment periods were excluded because study medication was not taken within 24 hours of assessment (one period of ASA 200 mg once-daily and one of ASA 100 mg twice-daily). The same 70 treatment periods were used for the secondary objectives, except for urinary dehydro-thromboxane B<sub>2</sub> analyses which were limited to the 67 treatment periods with the requisite data available.

### *Effect of ASA dose on COX-1 dependent tests*

Figure 1 shows the distribution of results for each COX-1 dependent platelet function test at baseline and for each of the three ASA doses. VerifyNow<sup>TM</sup>-ASA platelet reactivity decreased significantly from baseline  $650 \pm 19$  to  $448 \pm 68$  with ASA 100 mg once-daily,  $430 \pm 65$  with 200 mg once-daily and  $416 \pm 39$  with 100mg twice-daily ( $p$  all  $< 0.0001$ ). Modelled platelet reactivity was reduced to a greater extent with ASA 100 mg twice-daily versus 100 mg once-daily ( $p=0.043$ ), but not versus 200 mg once-daily ( $p=0.44$ ). ASA 200 mg once-daily did not differ from 100 mg once-daily ( $p=0.20$ ). Interestingly there were only 2 out of 24 patients who gave full aggregation responses by VerifyNow<sup>TM</sup>-ASA with ASA 100 mg once-daily. This was also mirrored in the other tests including serum TxB<sub>2</sub> and urinary dTxB<sub>2</sub>, PFA-100<sup>TM</sup>-CEPI and CADP but only 1 of the 2 still gave full aggregation with LTA-AA. Only 1 of these patients then gave a full response by VerifyNow<sup>TM</sup>-ASA with ASA 200 mg once daily but this was still consistent with serum TxB<sub>2</sub> and urinary dTxB<sub>2</sub>, PFA-100<sup>TM</sup>-CEPI and CADP and LTA-AA. However, at ASA

100 mg twice daily all platelet function was inhibited in this individual except PFA-100<sup>TM</sup>-CEPI. This could have been caused by true “ASA resistance” and/or poor compliance that was eventually overcome by ASA 100 mg twice daily. A failure to comply with the ASA dosage regime was felt unlikely in this individual as pill counts and interviews were performed and that both the serum TxB<sub>2</sub> and urinary dTxB<sub>2</sub> levels were still significantly reduced from baseline levels but above the accepted cut off values for these assays. The other COX-1 dependent platelet function tests also showed significant decreases from baseline with all three ASA regimens ( $p < 0.0001$ ). The generalized mixed model demonstrated that, compared to ASA 100 mg once-daily, ASA 100 mg twice-daily produced statistically significant reductions from baseline for all COX-1 dependent platelet function tests except LTA-AA. There was no statistical difference for COX-1 dependent tests between ASA 100 mg twice-daily and 200 mg once-daily. Furthermore, ASA 200 mg once daily more effectively reduced WBA-AA than 100 mg once-daily. Pharmacologic suppression of COX-1 activity compared to baseline was demonstrated at all ASA doses, as measured by decreased serum TxB<sub>2</sub> and urinary dTxB<sub>2</sub>. A greater pharmacologic effect was observed with ASA 100 mg twice-daily compared to 100mg once-daily for urinary dTxB<sub>2</sub> ( $p=0.048$ ) with a similar but non-significant trend for serum TxB<sub>2</sub>.

#### ***Effect of ASA dose on non-COX-1 dependent tests***

Figure 2 shows the distribution of results for each non-COX-1 dependent platelet function test at baseline and for each of the 3 ASA doses. Neither the PFA-100<sup>TM</sup>-CADP nor the WBA-ADP platelet function tests showed any significant reduction from baseline. ASA 100 mg twice-daily produced a greater effect on PFA-100<sup>TM</sup>-CEPI compared to 100 mg once-daily, but no such difference was observed with any other COX-1 independent test. There were no statistical differences for any COX-1 independent test between ASA 100 mg twice-daily and 200 mg once-daily.

#### ***Kappa Statistics***

The overall kappa statistic classifications for baseline and all ASA dosages are shown in Table 1. No differences were observed with each test for each ASA dose.

### ***Comparison with serum TxB<sub>2</sub>***

Figure 3 shows the scatter plots comparing each test with serum TxB<sub>2</sub> and shows the results for baseline and the 3 ASA dosage regimens. In general COX-1 dependent tests not only correlated better with serum TxB<sub>2</sub> than non-COX-1 dependent tests but gave better agreement.

### ***Comparison with LTA-AA***

Figure 4 shows the scatter plots comparing each test with LTA-AA and shows the results for baseline and the 3 ASA dosage regimens.

## **Discussion**

Full details of the primary outcomes of the trial have been previously published, showing that 100 mg of ASA twice-daily in T2D reduced platelet reactivity more effectively than 100 mg once-daily, and numerically more than 200 mg once-daily [15]. This regimen was also the most effective based on the pharmacologic effect on serum and urinary TxB<sub>2</sub> levels, and for reducing platelet reactivity as measured by a number of platelet function tests, *i.e.* VerifyNow<sup>TM</sup>-ASA, WBA-AA and PFA-100<sup>TM</sup>-CEPI.

We have now compared the secondary outcome results by comparing the performance of all nine different platelet function assays used in this trial. As this trial was a small study in 24 patients it was not feasible to measure the clinical efficacy of each test for prevention of ischaemic events. However, It is clear that the different platelet function tests evaluated are not equally effective in measuring the antiplatelet effects of ASA in T2D. The COX-1 dependent tests (e.g. VerifyNow<sup>TM</sup>-ASA, LTA-AA and Serum TxB<sub>2</sub>) in general not only performed much better in correctly identifying patients at baseline and on 3 different dosages of ASA (Figures 1 and 2) but also correlated better with serum TxB<sub>2</sub> levels and LTA-AA than non-COX 1 dependent tests (Figure 3). The incidence of ASA non-responders is therefore also much lower in COX-1 dependent tests at all doses of ASA. As all the assays used in this study measure different aspects of platelet function, the observed differences between the tests is unsurprising and confirms the results from previous studies comparing the results from these

tests in different types of patients receiving either ASA and/or P<sub>2</sub>Y<sub>12</sub> inhibitors [21-24]. One of the major limitations of these tests is the use of cut-off values that have not been clinically validated and are either arbitrarily defined or established upon the responsiveness of normal individuals to ASA either if given in vivo or added to blood in vitro. The VerifyNow™-ASA test is the only true FDA approved POC assay used in this trial and has been shown in some studies to relate to clinical outcomes [25, 26]. The advantage of this whole blood cartridge-based test is that it can be potentially used by non-platelet specialists away from the laboratory and does not require any pipetting of the blood sample during testing. As this test is now widely used, it is therefore important that the test is also validated against a range of tests as previous studies have shown variable results. In this study the overall agreement with either LTA-AA (k = 0.95, Figure 4) (the perceived gold standard) or Serum TxB<sub>2</sub> levels (k = 0.85, Figure 3) (a measure of pharmacological inhibition) was very good and it performed better than in other studies directly comparing these tests in different populations of patients [23, 24]. Given that the test now uses arachidonic acid, one would expect a reasonable correlation with other tests using the same agonists. The test was also performed in a specialist laboratory (and not as a POC test) at exactly the same time as other platelet function tests within locally taken fresh samples within a defined window of time and within a controlled clinical trial in a relatively homogeneous group of T2D patients. Some of the older comparisons of VerifyNow™-ASA were also performed using cartridges that utilised a different agonist (e.g. propyl gallate and not arachidonic acid) which may explain some of the previous reported differences [17, 27].

Of the other assays used in this study LTA-AA is still considered as the historical gold standard of platelet function testing [18]. As the assay also used arachidonic acid to drive platelet activation and aggregation it should be reasonably specific for measuring COX-1 activity as TxA<sub>2</sub> generation is a critical for amplification of initial platelet activation to drive secondary aggregation. Indeed, as one might expect LTA-AA not only agreed very well with serum TxB<sub>2</sub> measurements (k = 0.83) but also VerifyNow™-ASA (k = 0.95), the latter assay using the same agonist. However, despite its good performance in this study its major disadvantage is that it is a specialist test and therefore can only be used by operators with expertise in performing this test. We also used ADP as an agonist as this has been widely used to measure ASA responses by LTA. As ADP stimulation is not specific for measuring COX-1 activity, low

concentrations of ADP (< 10  $\mu$ M) still require COX-1 activity for full aggregation responses to occur. Higher concentrations of ADP (> 10  $\mu$ M) are thought to be COX-1 independent so there is a debate over which concentrations of ADP are optimal for measuring ASA inhibition. Indeed in this study we chose an intermediate concentration of 10  $\mu$ M ADP, as we have previously shown that ASA does inhibit secondary aggregation responses at this dose. It is probably not surprising that the overall performance was therefore moderate and with hindsight a lower concentration of 5  $\mu$ M ADP could have been used instead, which has been shown to perform better than higher concentrations for monitoring ASA [24]. As LTA is performed in PRP we also wanted to compare results with whole blood aggregometry as this is not only used in the VerifyNow<sup>TM</sup>-ASA test but is argued to be more physiological and does not require centrifugation steps to prepare samples for testing. WBA using the Multiplate system has also become an established test for monitoring P<sub>2</sub>Y<sub>12</sub> antagonists as this instrument uses disposable cuvettes and because of its ease of use has also been used as a POC test within intensive care settings [28]. In this study WBA using either AA or ADP as the agonists gave either a moderate or poor performance respectively. Although whole blood is probably more physiological the results are probably not surprising given that alternative mechanisms of platelet activation are highly likely to occur within whole blood which could potentially bypass any ASA inhibition of COX-1 activity. The PFA-100<sup>TM</sup> also utilises whole blood but does not measure platelet function in the same way as classical or whole blood aggregometry [19]. Instead, the instrument measures high shear dependent platelet thrombus formation in real time which could be argued to be more physiological and is rapid and simple to use. Again the test is FDA approved for measuring platelet dysfunction and has been widely used to detect ASA responses within the CEPI cartridge but not the CADP cartridge which has been previously shown to be relatively insensitive to ASA [29]. Indeed in this study the CEPI cartridge performed either moderately or good in comparison with ASA dose and serum TxB<sub>2</sub> measurements respectively. In contrast, the CADP cartridge performed poorly as expected. Previous studies have shown that the PFA-100<sup>TM</sup> CEPI cartridge can sometimes be totally insensitive to ASA and be effectively by-passed in conditions where there is platelet hyper-reactivity and/or high von Willebrand factor (VWF) levels, which are known to be elevated in patients with coronary artery disease or T2D [30]. This can even occur when COX-1 activity is effectively blocked by ASA as assessed by TxA<sub>2</sub> measurements in the

same samples. There was some evidence of normal closure times in this study particularly at the lower doses of ASA even though  $\text{TxA}_2$  levels suggested there was effective inhibition of COX-1. It could be that the VWF levels may have been higher in some individuals as this is an important variable for determining efficient platelet adherence to collagen and aggregation under the high shear conditions in the test. Unfortunately, VWF levels were not measured in this study but have been shown to contribute to normal closure times in other studies where poor responsiveness to ASA was observed [31].

As  $\text{TxB}_2$  is the major metabolite of platelet derived  $\text{TxA}_2$  in plasma/serum then this provides a direct and specific biochemical measurement of the effect of ASA on COX-1 activity. The presence of the thromboxane metabolite  $\text{dTx}_2$  in the urine can also be directly correlated with platelet activation and inhibition of COX-1 and thromboxane generation by ASA. In this study, however  $\text{dTx}_2$  only gave a moderate correlation with serum  $\text{TxB}_2$  levels. As  $\text{dTx}_2$  is being measured in urine from patients and unlike serum  $\text{TxB}_2$  is not being generated ex vivo, this provides a global measurement of  $\text{TxA}_2$  which could originate from other cellular sources as well as platelets [24]. Again these results are therefore unsurprising and are supported by previous studies that report discrepancies between platelet derived  $\text{TxB}_2$  and urinary  $\text{dTx}_2$ .

In summary in this study we have shown that not all platelet function tests used for monitoring ASA therapy in TD2 are equivalent and therefore the results are not interchangeable. As one might have predicted, platelet function tests that specifically measured COX-1 pathway (e.g. VerifyNow™-ASA, LTA-AA and Serum  $\text{TxB}_2$ ) were more sensitive in detecting ASA inhibition in general, although there were clear differences in responsiveness between different doses which is probably related to T2D and not the efficacy of the COX-1 dependent tests themselves.

Although routine ASA monitoring is not currently recommended, personalising ASA therapy in high risk patients (e.g. T2D) as shown in this trial may have potential benefit providing COX-1 dependent tests were used [15]. This would certainly have to be confirmed in large prospective studies to study clinical outcomes and whether there is any increased risk in bleeding with higher dosages of ASA especially over more prolonged periods of treatment. Current guidelines also suggest that  $\text{P}_2\text{Y}_{12}$  inhibitors should also not be monitored except perhaps again in high risk individuals, so any role for personalising antiplatelet therapy with

platelet function testing is still very uncertain. Future trials should also distinguish between measuring the *in vitro* efficacy of ASA versus clinical efficacy and prevention of ischemic events. It is likely that true “ASA resistance” is rare and often caused by poor compliance but underlying platelet hyper-reactivity can often increase the risk of thrombosis despite effective COX-1 inhibition by ASA [6, 32].

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## **Declaration of Interest Statement**

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## Figure Legends

**Figure 1.** Distribution of COX-1 dependent platelet function test results by each test for baseline (blue) and each ASA dose (100 mg (green), 200 mg (black) and 100 mg x 2 (red)). A – VerifyNow™-ASA (ARU), B – LTA (AA) (%), C – WBA (AA) (AUC), D – Serum TxB<sub>2</sub> (ng/ml) and E – Urinary dTxB<sub>2</sub> (pg/ml). Platelet reactivity was significantly decreased ( $P < 0.0001$ ) in all tests at each ASA dose compared to baseline. Any significant differences between ASA dosages in each test are shown.

**Figure 2.** Distribution of non-COX-1 dependent platelet function test results by each test for baseline (blue) and each ASA dose (100 mg (green), 200 mg (black) and 100 mg x 2 (red)). A – PFA-100™CEPI (seconds), B – PFA-100™-CADP (seconds), C – LTA (ADP) and D – WBA (ADP) (AUC). Dotted lines denote the cut-off values for each test. Platelet reactivity was significant decreased ( $P < 0.0001$ ) in the PFA-100™CEPI and LTA (ADP) tests at each ASA dose compared to baseline but not in the PFA-100™-CADP and WBA (ADP) tests. Any significant differences between ASA dosages in each test are shown.

**Figure 3.** Scatterplots comparing all platelet function tests with serum TxB<sub>2</sub> levels (ng/ml). Baseline values are shown as blue dots, 100 mg ASA as green squares, 200 mg ASA as black triangles and 100 mg x 2 ASA as red squares. A – VerifyNow™-ASA (ARU), B – LTA (AA) (%), C – WBA (AA) (AUC), D – Urinary dTxB<sub>2</sub> (pg/ml), E – PFA-100™-CEPI (seconds), F – LTA (ADP), G – PFA-100™CADP (seconds) and H – WBA (ADP) (AUC). The solid lines denote the cut-off values for each test.

**Figure 4.** Scatterplots comparing all platelet function tests with LTA-AA (%). Baseline values are shown as blue dots, 100 mg ASA as green squares, 200 mg ASA as black triangles and 100 mg x 2 ASA as red squares. A – VerifyNow™-ASA (ARU), B – LTA (AA) (%), C – Serum TxB<sub>2</sub> (ng/ml), D – Urinary dTxB<sub>2</sub> (pg/ml), E – PFA-100™CEPI (seconds), F – LTA (ADP), G – PFA-100™CADP (seconds) and H – WBA (ADP) (AUC). The solid lines denote the cut-off values for each test.

**Table 1.** Overall kappa statistic classifications for each platelet function test at baseline with either all or each individual ASA dosage regime.

Values 0–0.20 are classified as poor, 0.21–0.40 fair, 0.41–0.60 as moderate, 0.61–0.80 as good, and 0.81–1 as very good.

<b>ASA Dose</b>	<b>VerifyNow™- ASA</b>	<b>LTA- AA</b>	<b>WBA- AA</b>	<b>LTA- ADP</b>	<b>WBA- ADP</b>	<b>PFA-100™ CEPI</b>	<b>PFA-100™ CADP</b>	<b>Serum TxB<sub>2</sub></b>	<b>Urine TxB<sub>2</sub></b>
<b>all</b>	0.85	0.95	0.47	0.59	-0.07	0.56	-0.02	0.79	0.55
<b>100 mg OD</b>	0.91	0.96	0.41	0.49	-0.10	0.57	-0.08	0.74	0.62
<b>200 mg OD</b>	0.92	0.96	0.42	0.46	0.04	0.67	0.00	0.88	0.63
<b>100 mg BD</b>	0.96	1.00	0.42	0.46	0.25	0.79	0.04	0.96	0.67